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Characterisation of an Epidemic of Hepatitis A Virus Involving Intravenous Drug Abusers—Infection by Needle Sharing?

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An epidemic of hepatitis A virus (HAV) among intravenous drug abusers in Oslo involved 144 serologically confirmed cases. Another 26 patients (non-drug abusers), of whom 14 were derived from a single nosocomial outbreak, were associated with the epidemic. Sequencing of the VP1/P2A junction revealed that viruses associated with the epidemic were completely identical, whereas other HAV samples collected during the same period differed by up to 10 %. HAV was detected in the serum of 48 of 100 patients by a nested PCR. Viremia was observed as early as 25 days before the onset of clinical hepatitis, and up to 30 days after. The large number of patients within the drug abuser group, and the few secondary cases, raised the question of whether the virus could be transmitted by the use of needles. To establish whether viral contamination of drugs did contribute appreciably to maintaining the epidemic, we examined heroin and amphetamine confiscated during the period, using immunomagnetic separation coupled to nested PCR, but failed to detect any virus. Antibodies against hepatitis B virus and hepatitis C virus were common among the HAV infected drug abusers (43% and 81%, respectively), suggesting widespread sharing of needles. This observation and the large number of patients with a demonstrable viremia suggest that needle sharing may contribute to the dissemination of HAV. ***J. Med. Virol. 53:69–75, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: hepatitis A; molecular epidemiology; intravenous drug abuse; RT-PCR; narcotics

INTRODUCTION

The hepatitis A virus (HAV) is a non-enveloped, positive-stranded RNA hepatovirus within the Picornaviridae family [Hollinger and Ticehurst, 1996]. The virus is differentiated into seven genogroups, of which the first four are found in humans, genogroup I being the most prevalent [Lemon et al., 1992; Robertson et al., 1994; Hollinger and Ticehurst, 1996]. In Norway, hepatitis A is rare and usually associated with visits to endemic regions [Kielland and Siebke, 1991; Jensenius et al., 1997]. Recently, however, we observed several outbreaks among intravenous drug abusers, the largest occurring in Oslo in 1995–96. Similar outbreaks have been reported from other parts of the world [CDC, 1996].

The dominant mode of HAV transmission is presumably faecal-oral, typically by contaminated food [Mast and Alter, 1993; Hollinger and Ticehurst, 1996], but several reports have shown that blood is infectious by documenting parenteral transmission of HAV in relation to blood products [Robertson et al., 1994; Mannucci et al., 1994; Lawlor et al., 1996; Ruymann et al., 1996]. A study of experimentally infected non-human primates suggests the serum concentration of virus to be 2 to 3 logs below the concentration in faeces [Lemon, 1994].

The aim of the present study was to characterise the epidemic in Oslo. For this purpose we used both a diagnostic, nested reverse transcriptase-polymerase chain reaction (RT-PCR), designed for maximum sensitivity and based on a conserved region of the genome [Monceyron and Grinde, 1994], as well as an RT-PCR

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based on the more variable VP1/P2A junction, and coupled to sequencing suitable for characterising the genotype [Robertson et al., 1995].

We also wished to assess whether the intravenous injection of drugs may contribute to the dissemination of HAV. Recent epidemics in several cities in Scandinavia have affected primarily intravenous drug abusers [Jensenius et al., 1997]. As drugs reaching Europe may have been smuggled within the human gastrointestinal tract from countries where HAV is endemic, the possibility of viral contamination was considered. Furthermore, the reports demonstrating parenteral transmission of HAV suggest that the virus could be transmitted by needle-sharing. To evaluate this hypothesis, the frequency of demonstrable viremia in the patients was assessed, and viral indicators suggesting parenteral infections, i.e. hepatitis B virus (HBV) and hepatitis C virus (HCV), were examined.

MATERIALS AND METHODS

Samples

Serum samples were obtained from all the 144 reported cases of hepatitis A among drug abusers during the Oslo epidemic (August 1995 to June 1996), and from the 26 cases of hepatitis A among close contacts. Sera were also obtained from 36 cases of HAV in Oslo reported during the same period, but not associated with the epidemic, patients who presumably had contracted the virus abroad. More than one sample was available from several of the patients. The diagnosis of acute HAV infection was confirmed in all the cases by detection of IgM against HAV (Axzym HAV ABM, Abbott). Serological markers for hepatitis B virus were HBsAg and anti-HBc (Axzym HBsAg and CORE, Abbott) and for hepatitis C virus anti-HCV (Ortho HCV 3.0 ELISA). Serum samples were stored at −70°C. Aliquots of heroin (28 samples of 44 batches confiscated during July and August 1995) and amphetamine (19 samples of 49 batches confiscated in October and November 1995) were obtained from the Oslo police department (Kripos).

RT-PCR and Sequencing

RNA was isolated from 140 μl serum, using a Qiagen kit (QIAamp Viral RNA kit) following manufacturer's recommendations, and eluted in 50 μl of water. Aliquots (5 μl) were used immediately for RT-PCR and the remaining RNA stored at −70°C. For diagnostic screening of HAV we used a previously described nested RT-PCR based on a conserved region of the 3′ RNA polymerase gene [Monceyron and Grinde, 1994]. A database search was performed to confirm that the primers were functional with all presently known human HAV sequences.

For sequence analysis we designed a novel RT-PCR, based partly on the primers used by [Robertson et al., 1995], to analyse the VP1/P2A junction of the genome. The primers were: HAV6, 5′-TGTCTGGAGCACTGGATGG-3′ (2839–2857); HAV7, 5′-CATTTCAAGAGTCCACACACTTCT-3′ (3357–3380); HAV8, 5′-TGGTTTCTATTCAGATTGCAAATTA-3′ (2890–2914); and HAV9, 5′-TTCATTATTTCATGCTCCTCAGT-3′ (3264–3286). The numbers refer to the HM175 strain. The cDNA was transcribed from 5 μl RNA at 42°C for 30 min in 10 mM Tris pH 8.3, 50 mM KCl, 1 mM dNTP, 0.5 U/μl RNA-guard (Pharmacia), 0.1 U/μl Avian RT (Promega), 7.5 mM $MgCl_2$, 0.5 mM EDTA, 0.25 mM DTT and 0.625 μM specific primer (HAV7) at a final volume of 20 μl, followed by an enzyme inactivation step at 98°C for 5 min and 4°C hold. The outer PCR was run by adding 30 μl mixture to yield the following final concentrations: 50 mM KCl, 10 mM Tris pH 8.3, 2.7 mM $MgCl_2$, 0.25 mM primer HAV 6 and HAV 7, 1.25 U Taq and 0.4 mM dNTP. Three microliters of outer PCR products were transferred to the nested PCR. The conditions were the same except for the use of 2 mM $MgCl_2$ 0.25 mM dNTP and the addition of primers HAV8 and HAV9. Both PCRs were cycled (25×) at 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec in a Perkin Elmer 9600 thermal cycler. Defined concentrations of the HAV strain HM175 (kindly provided by Dr. S. Emerson, National Institute of Public Health, USA) were included as positive control. For negative controls we used both extracts from sera known not to contain HAV, and the addition of water instead of extract. The negative controls were always negative. The PCR products were visualised by UV-transillumination in 2% ethidium bromide stained agarose gels with a 123 bp ladder (Gibco BRL) as size marker. Products of the nested PCR were sequenced on a 310 ABI PRISM Genetic Analyser using the Dye Terminator Cycle Sequencing Ready Reaction Kit and the recommended protocol (PE Applied Biosystems). The results were confirmed by sequencing both strands using the two nested primers.

Examination of Heroin and Amphetamine

In order to extract virus from heroin and amphetamine immunomagnetic separation (IMS) was used [Monceyron and Grinde, 1994]. Briefly, 0.5 g aliquots were dissolved/suspended in either 1.5 ml water (heroin chloride) or 1.5 ml 50 mM acetic acid (heroin base). HCl or NaOH was used to neutralise the solutions as required, and buffer added to give 2 ml solutions containing 50 mM Tris HCl, pH 7.6, 50 mM KCl, 0.05% bovine serum albumin (IMS-buffer). The amphetamine was dissolved directly in the IMS-buffer with no neutralisation required. The preparations were vortexed twice for 30 sec and briefly centrifuged to sediment undissolved matter. One milliliter of each supernatant was transferred to new tubes along with 10 μl (100 μg) of magnetic beads (Dynal, Oslo, Norway) coated with monoclonal antibodies directed against a surface epitope of the viral particles. The tubes were kept on a rotator at room temperature for 1 hr. Subsequently a magnet was used to retain the beads (with captured viral particles) while discarding the supernatants. The beads were washed twice in IMS-buffer and a third time in PCR-buffer (50 mM KCl, 50 mM Tris HCl, pH 8.3). The washing procedures included 10 min

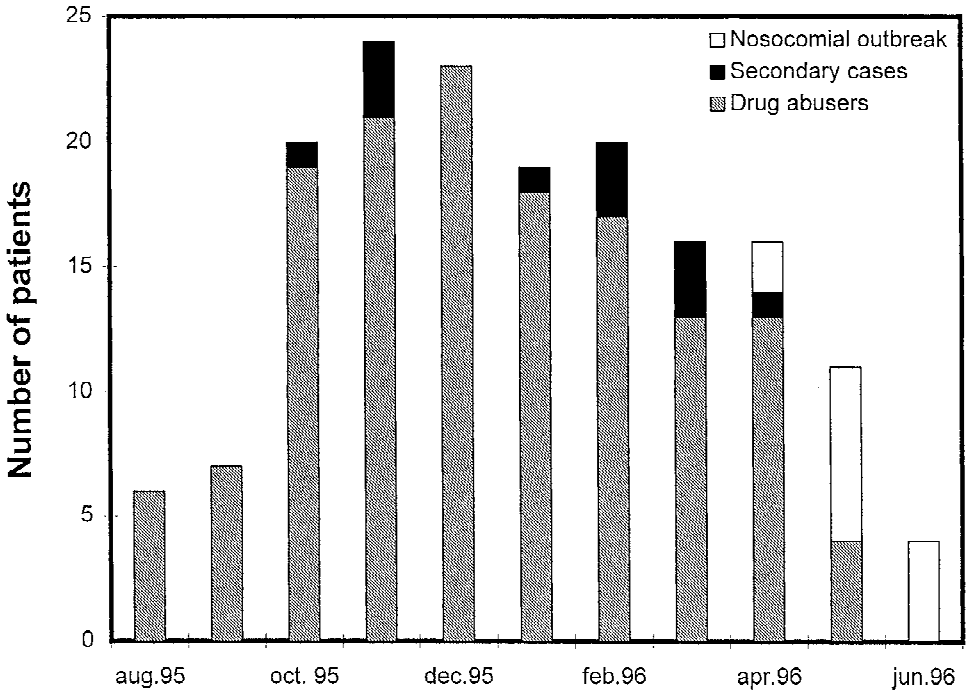

Fig. 1. Distribution of cases associated with the present HAV epidemic. The tips of the columns depict secondary cases, implying non-drug abusers who have been in contact with infected drug abusers. Patients belonging to a single nosocomial outbreak are included as an independent category.

incubations. The beads were finally dissolved in 5 μl of PCR-buffer and transferred to PCR tubes. The viral RNA was made available by heat-disrupting the viral particles (99°C, 5 min). The tubes were immediately transferred to ice and the diagnostic nested RT-PCR (2 × 35 cycles) started [Monceyron and Grinde, 1994].

RESULTS

Examination of Sera

The Oslo epidemic lasted from August 1995 to June 1996 and included 144 reported cases among drug abusers as well as 26 secondary cases among non-abusers, of whom 14 were derived from a single nosocomial outbreak (Fig. 1). A total of 196 serum samples from 100 of the drug associated patients were examined for the presence of HAV by a diagnostic RT-PCR. Of these, 56 samples from 48 different patients were positive. Of seven sera, taken up to 4 weeks prior to the onset of symptoms, one serum, taken 25 days before the onset of symptoms, was positive. Many more samples were available after the onset of clinical hepatitis. As shown in Figure 2, the majority of samples taken during the first week contained demonstrable HAV, and positive sera were observed as late as 30 days after the onset of illness. All samples (33) taken more than 4 weeks before symptoms or more than a month after onset (11 samples) were negative.

As shown in Table I, the prevalences of anti-HBc (43% positive) and anti-HCV (81% positive) among the present drug abuser patients were of the same magnitudes as in a study of intravenous drug abusers in Oslo from the late 1980s, but much higher than the prevalences found in blood donors or in persons referred to a clinic for sexually transmitted diseases. Nine patients (7%) had active HBV infection (HBsAg-positive), and four (3%) had acute hepatitis B. The secondary cases, i.e. HAV infected contacts of the drug abusers, were all negative for both HBV and HCV antibodies.

Sequence Analysis

In order to characterise the viruses 348 bp from the VP1/P2A region were sequenced. A total of 26 samples were sequenced, 12 from the outbreak and 14 from patients who presumably contracted the virus abroad. The 12 samples from the outbreak were identical. Among the other 14, two samples had sequences identical to those of the outbreak (Fig. 3, Spain and England1), but both were found retrospectively to be associated with the epidemic.

All the sequences clustered within genogroup I. A few cases imported from North Africa clustered in genogroup Ib, with less than 5 % variability (corrected distances based on the Kimura two-parameter method), while the other samples clustered in genogroup Ia, with 1–8% variability (Fig. 3). Sequence homology between genogroups Ia and Ib varied from 90 to 92%. Two cases from Morocco were 100% identical, as were two cases from Italy.

Attempts to Detect Virus in Confiscated Drugs

A total of 28 samples of heroin and 19 samples of amphetamine were tested for the presence of HAV by

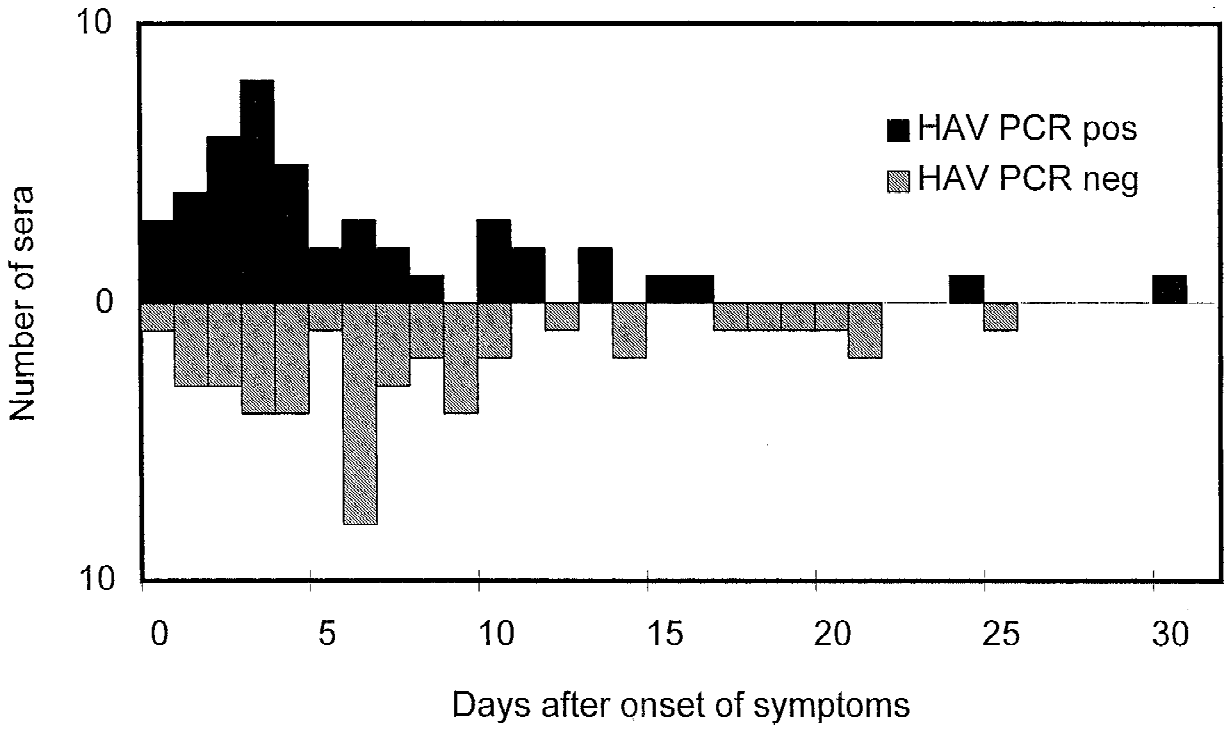

Fig. 2. HAV in sera as detected by the diagnostic PCR and related to days after onset of clinical hepatitis. Only sera from patients with known onset were included.

TABLE I. Examination of Viral Markers in Sera

	Indicators of infection[a]			
Group	Anti-HAV IgM	HAV PCR	Anti-HBc	Anti-HCV
Drug abusers	144/144	48/100	60/140 (43%)	101/125 (81%)
Related secondary cases	26/26		0	0
Unrelated cases	36/36	14/36[b]		
IV drug abusers			61/90[c] (68%)	68/90[c] (76%)
Patients of STD clinic			140/1829[d] (8%)	
Blood donors			5/1402 (0.4%)[e]	2/1402 (0.1%)[e]

[a]Examinations of the present HAV patients are indicated in the upper part, previous serological studies on populations in Oslo in the lower part of the Table.
[b]The VP1/P2A PCR.
[c]Intravenous drug abusers submitted to a clinic in 1985, 1988 and 1989 [Kielland and Siebke, 1991].
[d]Values from the unpublished sexually transmitted disease (STD) reflex study of 1989.
[e]Novel blood donors at the Ullevål Hospital Blood bank during 1992.

immunomagnetic separation (IMS) RT-PCR. In order to evaluate the method, aliquots of HAV (HM175) of known titters were seeded out in the various types of narcotics. As shown in Figure 4, the diagnostic, nested RT-PCR detected as little as 1,000 $TCID_{50}$ units per ml of HAV without using IMS to concentrate the virus. With IMS 1 unit per ml was detected when the virus was diluted in buffer, while when seeding the virus out in amphetamine, heroin chloride or heroin base, 10 units/ml was detected. All the samples of confiscated narcotics tested were negative.

DISCUSSION

Of the seven HAV genogroups, the vast majority of human strains are genogroup I [Hollinger and Ticehurst, 1996], and all the present sequences belonged to this genogroup. Sequences from patients associated with the epidemic were identical and clustered with group Ia, while group Ib sequences were found in a few patients believed to have contracted the virus in North Africa (Fig. 3). The strength of molecular epidemiology was revealed by the sequences obtained from two patients presumed to have been infected in Spain and England. Their sequences proved to be identical to those obtained from the present epidemic, and retrospectively both patients were found to be related to the epidemic, one as an intravenous drug abuser, the other as a nurse attending a patient from the epidemic. As they had both visited a foreign country during the period prior to onset of symptoms, they were originally placed among the unrelated cases.

Almost half the patients tested had at some point

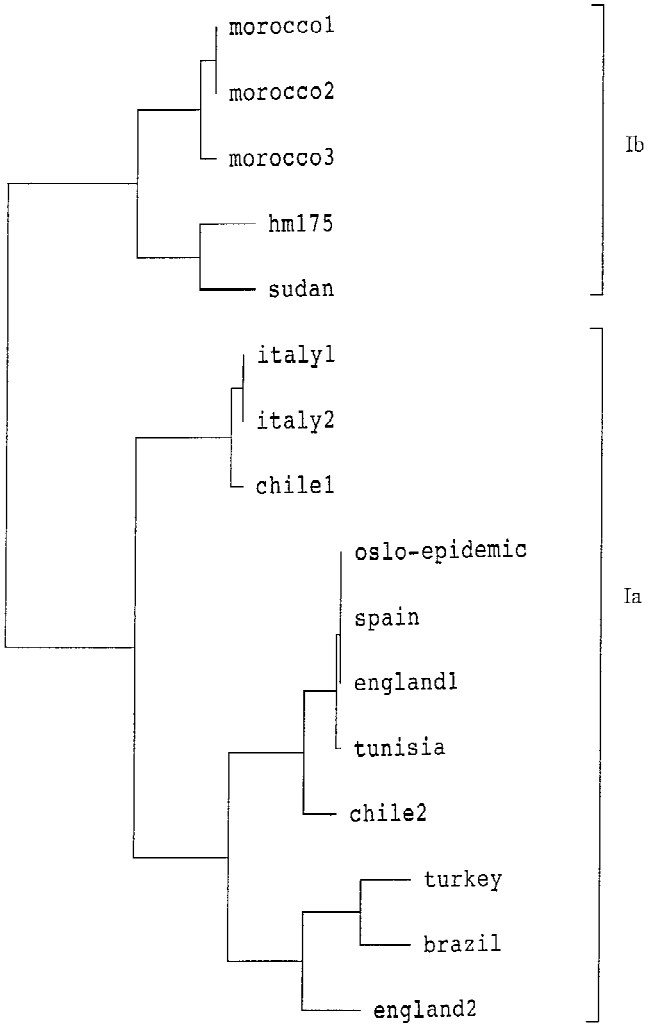

Fig. 3. Phylogenetic analysis of virus from the epidemic and unrelated cases reported during the same period. The unrelated sequences have been given names suggesting the country where the patients presumably contracted the virus. The well characterised HM175 strain is included as a control, and genogroups are indicated on the right hand side of the figure. The analysis was based on 348 bp from the VP1/2PA region, using the UPGMA method on Kimura two-parameter corrected distances.

HAV in their sera as detectable by RT-PCR (Table I). In cases where samples were obtained during the first week after onset of symptoms, the majority of serum samples proved positive (Fig. 2), implying that faecal samples may not be required to perform molecular epidemiology. The fact that all samples taken more than 4 weeks prior to the onset of clinical symptoms or more than a month after the onset of symptoms were negative strengthens the validity of the PCR. In a similar study, Yotsuyanagi et al. [1993] found HAV in 28% of the patients, but no later than 7 days after onset of symptoms.

We believe that it is preferable to employ two different PCRs when evaluating an epidemic. The diagnostic PCR was designed for optimal sensitivity and specificity, i.e. it used primers of relatively close proximity situated in a conserved region of the genome. In order to do epidemiology we chose a larger PCR product from a less conserved region (VP1/2PA) and ran fewer cycles. Of 48 samples positive in the diagnostic PCR, 42 were positive in the PCR designed for epidemiology. Of 36 cases not related to the epidemic, 14 were positive in the latter PCR.

The principal mode of transmission of HAV is by the faecal-oral route [Hollinger and Ticehurst, 1996]. If this were the only route of infection, more secondary cases among non-drug abusers might have been expected. Of a total of 26 such patients, only eight cases were family and friends, the remainder being health workers and fellow-patients associated with nosocomial outbreaks.

Recently the transmission of HAV by blood products was demonstrated [Robertson et al., 1994; Mannucci et al., 1994; Lawlor et al., 1996; Ruymann et al., 1996]. The PCR results, suggesting that viremia may last for up to 2 months, and that virus can be demonstrated in most patients if samples are obtained during the first week after onset of symptoms, even though the peak viremia occurs prior to the onset of symptoms [Yotsuyanagi et al., 1993; Lemon, 1994], may suggest that the potential spread of virus by contaminated blood is a more serious problem than recognised previously.

When asked, the present drug-abuser patients tended to deny sharing of needles, and the low prevalence of HIV in this group [Aavitsland et al., 1996] suggests that the addicts are able to avoid needle sharing with those harbouring HIV. However, the high prevalences of HBV- and HCV-markers indicate that needle-sharing is common (Table I). It is therefore conceivable that HAV was transmitted by the sharing of needles in the present epidemic. Considering this virus, as well as the high prevalence of HBV and HCV, it may be relevant to further stress for the drug abusers that the sharing of needles is unsafe even in the absence of HIV.

The observation that recent epidemics of HAV in Scandinavia have centred around the intravenous drug abusers [Jensenius et al., 1997] prompted us to investigate narcotics as a possible vehicle of transmission. As narcotics are often smuggled in the intestinal tract from countries endemic to HAV, virus could conceivably be present in the drugs. If viral contamination had been a major problem, HAV would have been detected in some of the samples. As no such viruses were found, it is considered unlikely that the present epidemic, lasting for almost a year, was maintained by contaminated drugs. The initial case(s) may, however, have contracted the virus in this way.

The present epidemic was considered a significant health problem for the community. One patient died, seven developed severe, but reversible, hepatitis, and 64 patients were hospitalised for a total of 504 days [Jensenius et al., 1997]. In order to limit the epidemic among the estimated 3,000 intravenous drug users in Oslo, the health authorities provided the high risk groups with gamma globulin (a total of 140 doses, until December 1995) and with vaccination using a total of 420 doses of Havrix 1440 (from December 1995 to May

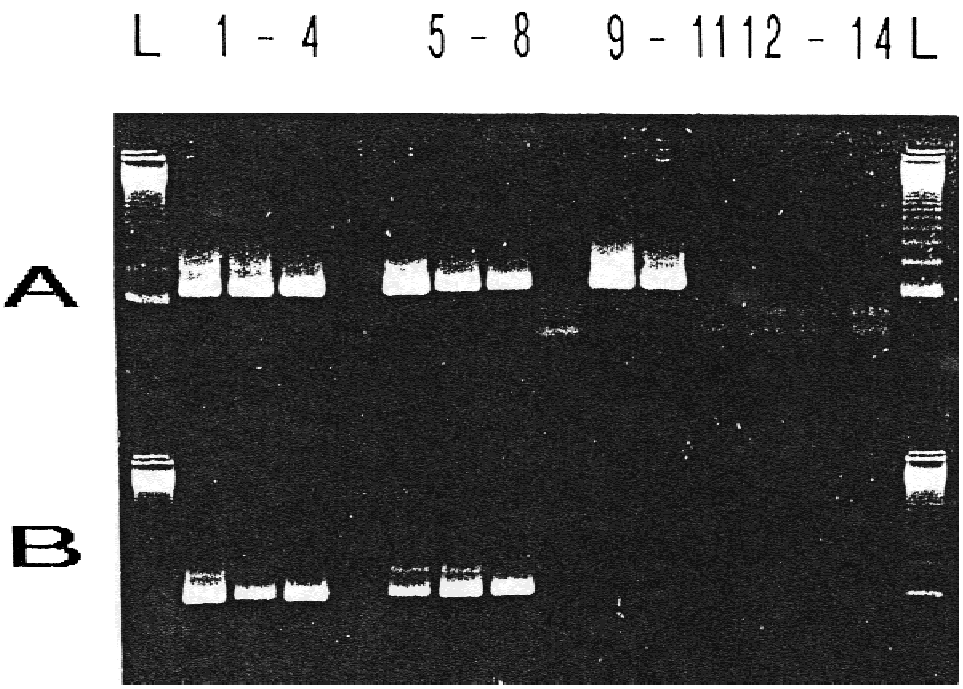

Fig. 4. Detection of HAV in amphetamine and heroin by IMS-RT-PCR. Tenfold dilutions of the HM175 strain (the concentration is given as the $TCID_{50}$/ml and denoted virus/ml) were analysed either directly by the diagnostic, nested RT-PCR (10^5–10^2 virus/ml, lanes 1–4 in **A**); or by IMS-RT-PCR after being seeded out in either IMS-buffer (10^2–10^{-1} virus/ml, lanes 5–8 in A); amphetamine (10^2–10^0 virus/ml, lanes 9–11 in A); heroin chloride (10^3–10^0 virus/ml, lanes 1–4 in **B**; or heroin base (10^3–10^0 virus/ml, lanes 5–8 in B). Representative results of analysing confiscated narcotics are shown: amphetamine, lanes 12–14 in A; heroin chloride, lanes 9–11 in B; and heroin base, lanes 12–15 in B. The products are visualised by ethidium bromide staining. L = 123 bp ladder. The main band (154 bp) is the inner PCR product, the larger band (343 bp) is the outer PCR product. Primer artefacts are visible in the negative samples.

1996), as well as information on hygiene and possible risk factors. The prophylaxis was offered free of charge.

The vaccine has been shown to limit epidemics [Werzberger et al., 1992; Prikazky et al., 1994]. As shown in Figure 1, the number of cases per month did decline after December 1995. A comparable epidemic among intravenous drug abusers in Malmö-Lund in Sweden lasted for almost 2 years. In this case neither gamma globulin nor vaccines were employed for prophylaxis, suggesting that the present strategy did help to confine the virus [Jensenius et al., 1997].

In the years prior to the present epidemic approximately 40 cases of hepatitis A were reported each year in Oslo, but the majority of these patients presumably contracted the virus abroad [Hasle and Espinoza, 1995]. In 1985, however, there was a similar outbreak among intravenous drug abusers including 120 reported cases [Jensenius et al., 1997]. During the 10 years that passed between the two epidemics, a novel population of abusers without antibodies against the virus were probably recruited.

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